Am. J. Trop. Med. Hyg., 106(1), 2022, pp. 222–228
doi:10.4269/ajtmh.21-0697

Molecular Identification of the Human Pathogen *Amphimerus* sp. in the Freshwater Snail *Aroapyrgus* sp. in Ecuador

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Abstract. Here, we report for the first time the snail intermediate host for the *Amphimerus* liver fluke, a foodborne trematodiasis. In Ecuador, *Amphimerus* of the Opisthorchiidae family, infects humans, cats, and dogs, in the tropical Pacific-coast region. Opisthorchiidae comprising also *Clonorchis sinensis*, *Opisthorchis* sp., and *Metorchis* sp., have complex life cycles involving a definitive and two intermediate hosts. We identified morphologically and investigated the presence and prevalence of *Amphimerus* cercaria and DNA in freshwater snails collected in a human-amphimeriasis endemic region in Ecuador, extracted DNA from snail tissue and emerged cercariae, performed real-time polymerase chain reaction (PCR) with the newly developed primers and probe amplifying the *Amphimerus* ribosomal internal transcribed spacer 2 (ITS2) region, and sequenced the amplified DNA fragment. We collected 2,800 snails, characterized four species *Aroapyrgus* sp., *Melanoides tuberculata*, *Biomphalaria cousini*, and *Aplexa marmorata*, isolated three cercariae morphotypes. Of the 640 snails analyzed by qPCR, only *Aroapyrgus* and one of the three cercariae resulted positive, at a 15% infection prevalence. Polymerase chain reaction revealed that the *Aroapyrgus* snail and cercaria-morphotype-3 corresponded to *Amphimerus*, but not to *C. sinensis*, *Fasciola hepatica*, or *Paragonimus mexicanus*. The sequence of amplified DNA product matched that of human-isolated *Amphimerus*. This finding constitutes the first documentation that *Aroapyrgus* sp. is the first intermediate host for the *Amphimerus* sp. that infect humans in Ecuador. The ITS2–gene PCR and sequencing analysis demonstrated a high prevalence of snail infection and proved useful for detecting the infection in snails, which findings can help the establishment of suitable control programs against transmission in any endemic region of interest.

INTRODUCTION

The genus *Amphimerus* Barker 1911 belongs to the Platyhelminthes, Trematode, Digenea, and Opisthorchiidae family. According to the WHO, the genera *Clonorchis*, *Opisthorchis*, and *Metorchis* of the Opisthorchiidae family are considered pathogens producing neglected tropical diseases within the category of foodborne trematodiases.[1] Foodborne trematodiases have been reported from more than 70 countries worldwide with Asia and Latin American countries being the most affected. Estimates referring to a selected group of 17 countries indicated that more than 56 million people were infected, of whom 7.9 million suffered severe sequelae and more than 7,000 died. Foodborne trematodes cause infection in humans via the consumption of contaminated food (raw fish, crustaceans, or vegetables). Infection can result in severe liver and lung disease, where together those pathologies are estimated to cause 2 million life years lost to disability and death worldwide every year.[1]

The genus *Amphimerus*, of cosmopolitan distribution, has been described in the bile ducts of mammals, birds, and reptiles.[2] Recently, a high frequency of infection was reported in humans and in domestic dogs and cats from Ecuador,[3–6] with the prevalences in humans being 24–36% within different locations of the rural tropical Pacific coastal region.[3,5,6] In addition, the prevalence of infection was 71.4% in cats and 38.7% in dogs.[4] Ecuador is a country located in the northwest of South America, under the Equator, crossed by the Andes that divided into three geographical regions: the Andean temperate region, the Pacific-coast tropical zone, and the interior tropical Amazon basin. The population living in the rural Coastal region comprises 2,254,145 inhabitants (https://www.ecuadorencifras.gob.ec/proyecciones-poblacionales/).

Opisthorchiidae trematodes have a complex life cycle with at least two intermediate hosts. Adult flukes reside in the biliary ducts of the definitive host (e.g., certain mammals); eggs are then discharged and shed in the stools to contaminate the freshwater of rivers, streams, lakes, and ponds. The eggs must be ingested by a suitable snail that acts as the first intermediate host, in which eggs hatch to release miracidia, where the latter go through the three developmental stages constituting sporocysts, rediae, and cercariae. The cercariae are released from the snail and, after a short time of free swimming in water, encounter and penetrate the flesh of freshwater fish (the second intermediate host), in which they encyst as metacercariae, the infective stage. Infection of humans and other definitive hosts occurs by ingestion of raw or undercooked freshwater fish.[7]

Although the freshwater snail hosts of *Clonorchis sinensis* and *Opisthorchis* spp. have been widely studied, the first intermediate hosts of *Amphimerus* sp. are still unknown in tropical regions endemic for human infections.[3,6] The snails identified as first intermediate hosts for *C. sinensis* are *Parafossarulus manchouricus*, *P. anomalospiralis*, *Alocinma longicornis*, *Bithynia fuchsiana*, *B. misella*, *Melanoides tuberculata*,

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Semisulcospira libertina, *Assiminea lutea*, and *Tarebia granifera*; for *Opisthorchis viverrini* are *Bithynia (siamensis) goniomphalus, B. (siamensis) funiculata,* and *B. (siamensis) siamensis*; for *Opisthorchis felineus* are *Bithynia leachii*, *B. inflata*, and *B. tentaculata*; and for *Metorchis conjunctus Amnicola limosus*.[8] For *Amphimerus elongatus* the only report involves a Hydrobiid snail identified as *Amnicola limosa* (Say), collected from Half Moon Lake, Eau Claire, WI.[9]

The identification of intermediate hosts and their respective liver fluke species in endemic regions are relevant to public health policies. Opisthorchiidae species are zoonotic, which is a concern for the resident potential-host population as well as for visitors. Therefore, the objective of this study was to discover the freshwater snails implicated in the transmission of *Amphimerus* in areas previously identified as endemic for human amphimeriasis, using molecular genetic tools. In the work reported here, for the first time we incriminate and determined the prevalence of the freshwater snail *Aroapyrgus* sp. as the first intermediate host for *Amphimerus* after molecular assaying the isolated larvae (cercariae) and the parasites in entire snails collected from an area in Ecuador known to be endemic for human amphimeriasis.

MATERIALS AND METHODS

Study area. Ecuador is located in the northwest coast of South America on the Equator. The Andes range divides the country in three natural regions: in the west, the tropical coastal region limited by the Pacific Ocean, in the center the temperate Andean region, and into the east the tropical rainforest of the Amazon basin. Within the coastal region, the study site—that is, the endemic area—lies within the Manabí province, and the Jipijapa Canton, Pedro Pablo Gomez (PPG) parish (−1.6266027595199972; −80.56018349758237) about 495 m above sea level. The distance from Quito, the capital of Ecuador, is 469 km. The streams surveyed were those that cross the rural villages studied in our previous publication and considered endemic for human and animal amphimeriasis.[6] The streams are part of the PPG River system, some with permanent water throughout the year. Numerous reservoirs are also present with relatively appropriate environmental conditions for providing suitable habitats for freshwater snails (Figure 1).

Snail sampling. Snails were collected at three sites on the PPG River during the rainy and dry seasons of the years 2019 and 2020. All the freshwater snails were collected from the borders and center zones of the streams with slow flowing or standing water and from nearby ponds. The snails were sampled by using a standard flat wire mesh scoop.[10] The snails collected from each stream were placed separately in plastic screw-capped containers and transferred alive to the Laboratory of Research of the Universidad de las Americas in Quito.

Morphological identification of snails. The snails were identified at the family, genus, and species levels using taxonomic keys.[11–13] Snails were immersed for 40 sec in water heated at 70°C and then transferred directly to water at room temperature. The soft parts were detached from the shell with forceps applied to the cephalopedal mass and fixed in 70% (v/v) aqueous ethanol. The shells were cleaned with a solution of bleach. The soft parts preserved in ethanol were dissected under a stereoscopic microscope and the reproductive system analyzed.

Cercarial shedding. Each snail species was separated in groups of five individuals for the cercarial-emission method.[14] Of the 1,840 specimens of *Aroapyrgus* sp. collected, 200 were placed for cercarial shedding along with 100 of the 920 of *Melanoides tuberculata* plus all 20 of the *Biomphalaria cousini* and *Aplexa marmorata* snails. Each group was separated in a sterile six-well culture plate (Thermo Scientific, Inc., Waltham, MA) filled with 5 mL distilled water and small pieces of lettuce leaves. For each group, the cercarial emissions were monitored for over 5 days with the water changed every day. The snails were exposed to an artificial bright light (25-W) source at 30 cm for 3 hour/day at room temperature (12–20°C) during which the shedding took place. Each well was checked for the presence of cercariae under a stereoscopic microscope. Individual cercariae shed in the collection of five snails were separated for morphological identification in a 1.5-mL tube containing saline solution and labeled according to the source. Once the shedding period was over, the snails were stored in 70% ethanol until subsequent molecular-genetic analyses.

Morphological identification of trematode cercariae. The identification of trematode cercariae was performed in a few drops of water on a glass slide, covered with a cover slide, and then inspected under an optical microscope (×40) as described by Frandsen F and Christensen NØ.[15] The cercariae of selected morphotypes were transferred to a glass slide containing 10% (v/v) aqueous formaldehyde, stained with the Diff-Quik® reagent (SYSMEX, Kobe, Japan), and identified at the trematode level through the taxonomic keys.[16–18] Cercariae morphotypes were photographed with a digital camera (Olympus, Tokyo, Japan) adapted to a biological microscope (Olympus CX-43, Tokyo, Japan). The rest of the individual cercariae were preserved in 70% ethanol for subsequent molecular-genetic analysis.

DNA extraction from snails, cercariae, and adult worms of *Amphimerus* sp., *Clonorchis sinensis, Paragonimus mexicanus*, and *Fasciola hepatica.* The DNA extraction was performed following the Chelex-100 method reported by Suenaga and Nakamura[19] with some modifications depending on the type of sample. The procedure stated in brief: 1) For cercariae, each individual of the three morphotypes selected were placed in a separate 1.5-mL microtube; 2) for adult trematodes, a piece of 2 mm^2 was cut and placed in a 1.5-mL microtube; and 3) for each snail, the entire soft tissue from *Aroapyrgus* sp. ($N = 500$), *M. tuberculata* ($N = 100$), *B. cousini* ($N = 20$), or *A. marmorata* ($N = 20$) was deposited in a separate 1.5-mL microtube. Before transfer to the tube, however, the tissue was crushed with a sterile pestle and then likewise after the first incubation. Then 200 μL of 10% Chelex resin (w/v; Sigma-Aldrich. St. Louis, MO) and 5 μL of proteinase K (Invitrogen, Carlsbad, CA) were added. The samples were incubated at 56°C for 60 minutes then vortexed and centrifuged at 8,000 g for 2 minutes. The resulting supernatant and pellet were incubated at 96°C for 20 minutes before a second centrifugation at 8,000 g for 5 minutes and transfer of the supernatant to a fresh microtube. All the samples were finally stored at −20°C for future use. Adult *Amphimerus* parasites were obtained from Ecuadorian patients as previously described,[3] *Clonorchis sinensis* was obtained from Japan by H. S., *Fasciola hepatica* and *Paragonimus mexicanus* worms were obtained from cattle of the

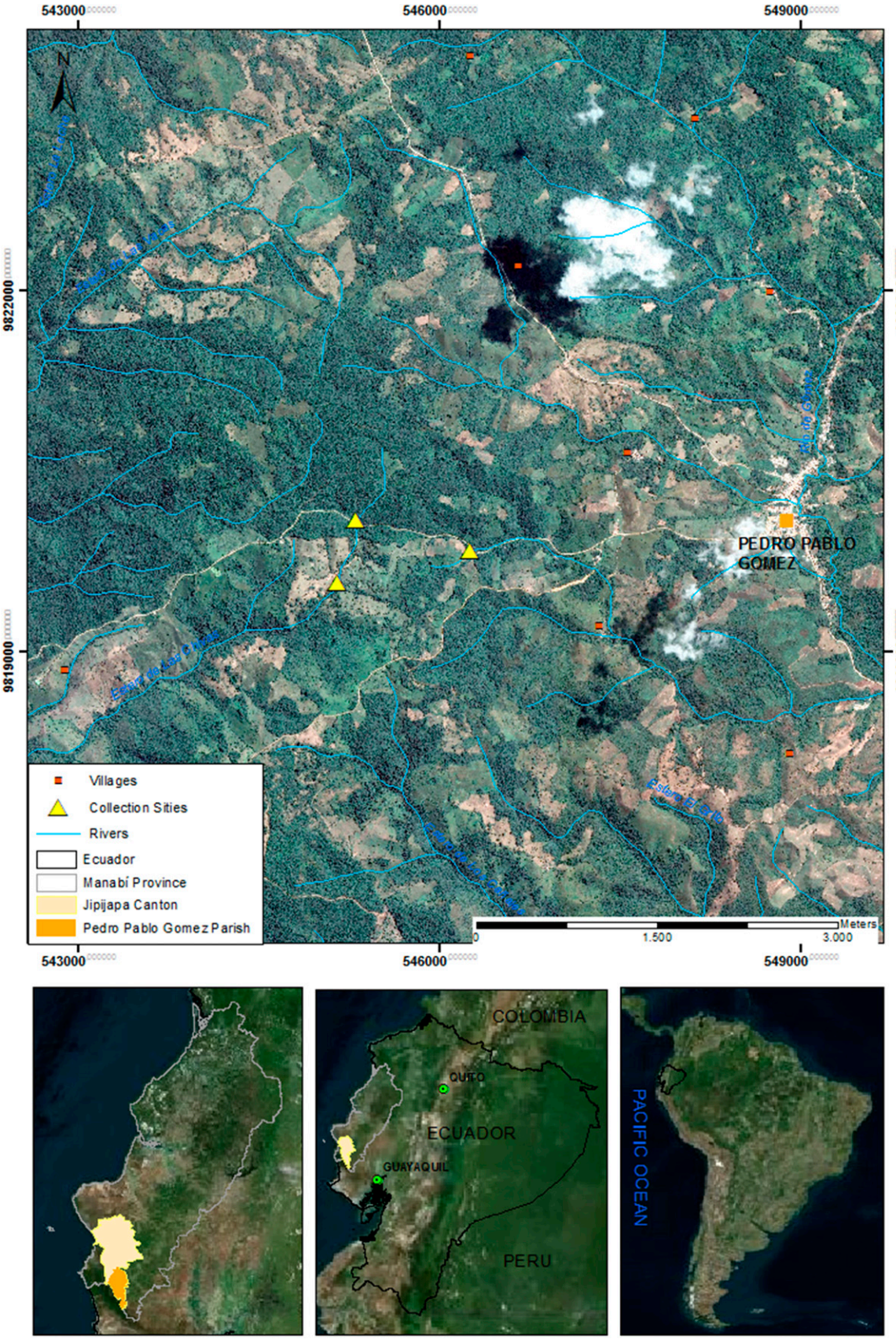

FIGURE 1. Map of Ecuador indicating the country's location on the northwest coast of South America (the entire continent pictured in right *inset* below) with an enlargement of the study area in the left *inset* below along with the sites for snail collection (yellow triangles in the central map illustrating the sampling sites). The streams flow into the Pedro Pablo Gomez (PPG) river which water body, in turn, flows into the Pacific Ocean. Most of the villages (small red squares) are located alongside the streams. The maps were developed from the shapefiles at https://www.naturalearthdata.com/. This figure appears in color at www.ajtmh.org.

Ecuadorian highlands and from an infected patient of the Pacific Coastal region, respectively.

Real time-polymerase chain reaction for detection of *Amphimerus*-specific DNA. The molecular-genetic identification of all three *Amphimerus* samples (adult parasite, cercariae, and naturally infected snails) was performed by the real-time polymerase chain reaction (RT-PCR) technique with the GoTaq Probe qPCR Master Mix Kit (Promega, Madison, WI). The primers and probes were designed for this specific research in targeting the internal transcribed spacer 2 (ITS2) region (GenBank/EMBL/DDBJ, Accession number AB678442). The reaction mix contained 1X of GoTaq probe qPCR Master Mix, 0.9 μM of each primer (AmphiN-F 5′-TCTATGGCTTTTCCCCAATG-3′ and AmphiN-R 5′-CGAGGTCAGGAAAGTTGAGC-3′), 0,25 μM of the probe (amphiprobe 5′-6-FAM-TGTTGTGACTATGCGCGCTCCGTTA-BHQ1-3′), and 2 μL of a 10 ng/μL DNA solution in a final reaction volume of 10 μL. The PCR amplification was performed in a thermocycler Biorad CFX96 (BioRad Inc., Montreal, Canada) with an initial denaturation step of 95°C for 2 minutes, followed by 45 cycles of a second denaturation step at 95°C for 15 seconds plus an annealing step at 62°C for 1 minute. The results were analyzed by the software Biorad CFX maestro TM software v1.1. DNA extracted from the adult trematodes of *C. sinensis, P. mexicanus*, and *F. hepatica* were used as specificity controls. Positive and negative controls were performed by mixing DNA from a human adult *Amphimerus* worm and extracted DNA from previously confirmed noninfected *Aroapyrgus* snails, respectively.

DNA Sequencing and the phylogenetic tree analysis. *Amphimerus* PCR–positive samples from both cercariae and the snail tissue were sequenced. The amplification products were purified before sequencing. The procedure stated in brief, Sanger sequencing was performed in an ABI 3500xL Genetic Analyzer (Applied Biosystems, Bedford, MA) with a BigDye 3.1® capillary electrophoresis matrix. The sequences obtained were compared with the genetic sequences in the GeneBank database and were submitted for acquisition of an accession number. Finally, DNA sequence analysis were performed in MEGA X.

Ethics. Research protocols were approved by the Ministry of the Environment of Ecuador (MAE), (Contract number MAE-DNB-2018-0090).

RESULTS

Snail collection and identification. In total, we collected 2,800 snails. Four species were morphologically identified through shell and anatomical characters: *Biomphalaria cousini* ($N = 20$), *Aplexa marmorata* ($N = 20$), *Melanoides tuberculata* ($N = 920$), and *Aroapyrgus* sp. ($N = 1,840$), belonging to the Planorbidae, Physidae, Thiaridae, and Cochliopidae families, respectively (Figure 2).

Cercarial shedding. Three morphotypes of cercariae were observed, referred to as morphotypes 1, 2, and 3 (Figure 3). *Aroapyrgus* sp. shed all three cercarial types, whereas *M. tuberculata* shed types 1 and 2. No cercariae were released by *A. marmorata* or *B. cousini*. Based on the morphologic stereomicroscopical characteristics of the cercariae that we obtained, we provisionally identified morphotype-3 as being *Amphimerus* sp. and the other two types as unrelated species.

Molecular genetic identification of *Amphimerus* DNA from adult trematodes, cercariae, and within snails. The amplification specificity was evidenced through obtaining positive results in the three samples of *Amphimerus*: the adult flukes, the cercariae, and the naturally infected snails. The other closely related trematodes—*C. sinensis, P. mexicanus*, and *F. hepatica*—resulted negative (Supplemental material). *Aroapyrgus* snails were positive at an incidence of 15% (72/500) for the amplification of *Amphimerus* DNA. All *M. tuberculata* ($N = 100$), *B. cousini* ($N = 20$) and *A.*

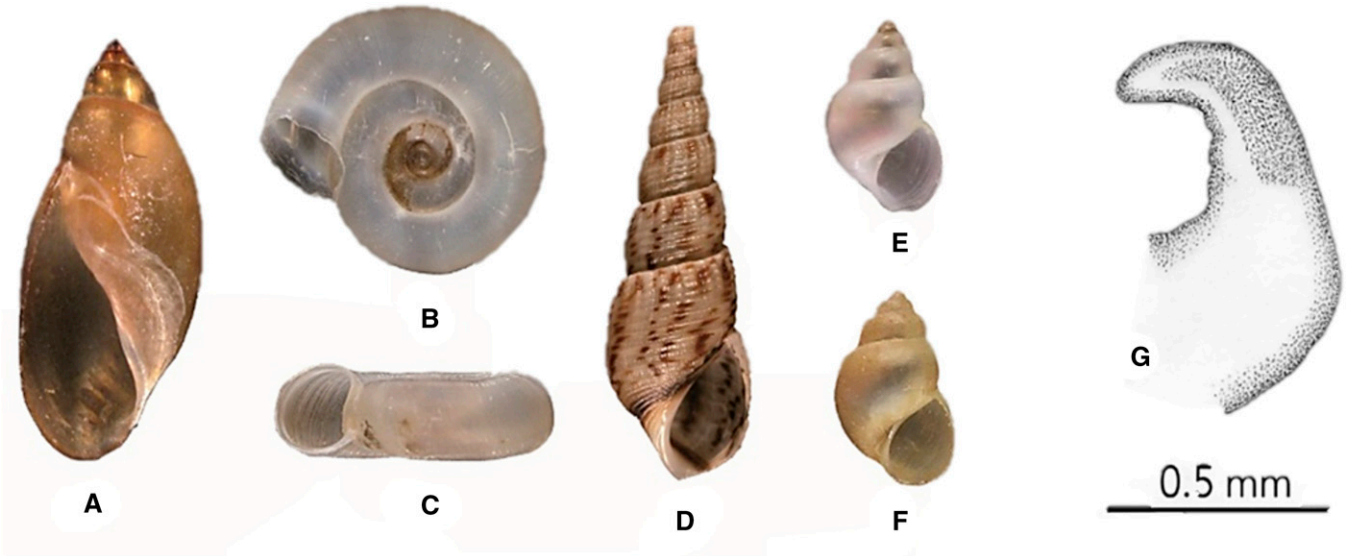

FIGURE 2. Photographs of the shells of the four snails identified in the Pedro Pablo Gomez (PPG) River of Ecuador: (**A**) *Aplexa marmorata*, H = 10.2 mm; (**B** and **C**). *Biomphalaria cousini*, D = 10.6 mm; (**D**) *Melanoides tuberculata*, H = 25.2 mm; (**E** and –**F**). *Aroapyrgus* sp. with and without the periostracum, H = 4.1 mm; and (**G**) dorsal view of the anatomy of the penis of *Aroapyrgus* sp. In the Cochliopidae family, the anatomy of the male penis is considered as a distinctive character at the genus level. Contrary to the other Cochliopid genera in the genus, the penis of *Aroapyrgus* sp. is simple without papillae. (H = shell height; D = diameter). This figure appears in color at www.ajtmh.org.

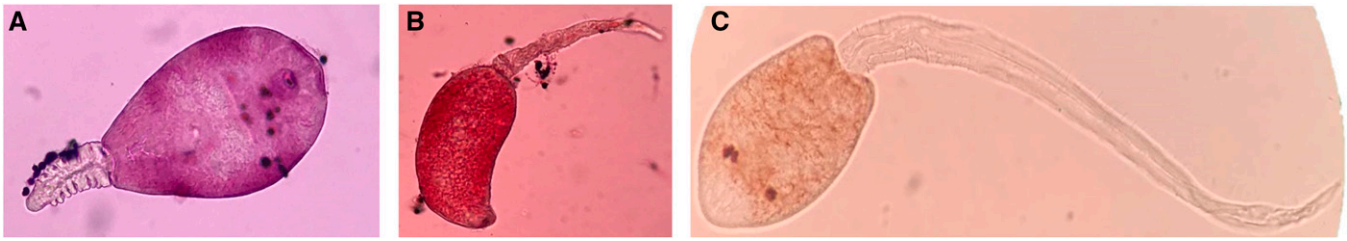

FIGURE 3. Optical microscopy of cercariae morphotypes found in *Aroapyrgus* snails after the cercarial-shedding method. (**A**) type 1; (**B**) type 2; and (**C**) type 3 (40× magnification). In general, all three types presented a body plus a tail, with the body measuring between 0.220 mm and 0.240 mm in length and 0.060 mm and 0.090 mm in width. All three presented oral suckers and acetabula, the acetabulum in each lay between the penetration glands and the excretory vesicle. A pair of eyespots were present in the anterior third of the body. The body surface was covered with microscopic spines. Morphotype 3 presented a tail at double the length of the body, measuring 0.370–0.480 mm and covered with transversely lined transparent cuticula over the whole surface, a fin-like membrane can be seen dorsoventrally. The tails were not bifurcated. This figure appears in color at www.ajtmh.org.

marmorata ($N = 20$) snails were negative for *Amphimerus* DNA. The sequence analyses of positive *Aroapyrgus* snails and cercaria type 3 confirmed the presence of *Amphimerus* in *Aroapyrgus* snails. The amplification products for *Amphimerus* (one from isolated cercariae and the other from infected *Aroapyrgus* snails) were sequenced and registered in GenBank with accession numbers MW411333 and MN512335, respectively. The phylogenetic analysis of the sequences obtained in this study confirmed the similarity to the sequences of previous studies of human *Amphimerus* metacercariae (AB678442). Furthermore, the proximity of other related trematode sequences such as *C. sinensis* (MK179281), *P. mexicanus* (LC317061), and the *F. hepatica* out group (AM8550107) was confirmed by the maximum-likelihood method based on the Jukes-Cantor model (Supplemental material).

DISCUSSION

In the work reported here, we identified for the first time the freshwater snail *Aroapyrgus* sp. as the first intermediate host in the life cycle of *Amphimerus* sp., the liver fluke that infects humans, cats, and dogs in an endemic area within the rural tropical coastal region of Ecuador. A comprehensive knowledge of the snail hosts implicated in this parasite's life cycle is essential for understanding the transmission and epidemiology of the disease to implement effective control strategies.

The high prevalence of *Aroapyrgus* infection with *Amphimerus* found in this study of 15% (72/500 snails) is worrisome, but not surprising because the prevalence of infection reported in our previous study in humans in those same rural communities was 36%, as was likewise found in dogs and cats.[6] These data determine that the region studied is highly endemic in *Amphimerus* transmission, which pathogen is dynamic throughout the year. Therefore, in view of the high prevalence of parasitism in this gastropod, *Aroapyrgus* is evidently a highly efficient intermediate host in the life cycle of this liver fluke, whose endemicity is of great significance with respect to transmission to humans, domestic and wild mammals, and piscivorous avians. The large population living in the rural coastal region are at risk of being infected if they consume raw or undercooked freshwater fish.

Because of the observed conchologic characteristics and the anatomy of the penis, the host snail clearly belongs to the genus *Aroapyrgus*. The genus *Aroapyrgus*, Cochliopidae, is a New-World group that is widely distributed in the neotropical area. A total of 30 species have been described from Mexico and from several countries ranging from Central America to South America including Venezuela, Colombia, and Brazil.[20] Notwithstanding, this group of small freshwater snails currently needs a stringent scrutinization by molecular-genetic techniques to clarify the systematic position of the different species reported among this neotropical genus. Therefore, further phylogenetic studies are required.

The identification of *Amnicola limosus*, a Hydrobiid snail, as the first intermediate host for *Amphimerus elongatus* in Half Moon Lake of Wisconsin,[9] where no human cases have been reported, implies the possibility that *Amphimerus* may infect other snail species. Hydrobiidae, commonly known as mud snails, are found in much of the world.[21] This prevalence could explain the worldwide distribution of *Amphimerus* reported in other animals and fish-eating birds.[2] *Amnicola limosus* is also implicated in the transmission of the Canadian liver fluke *Metorchis conjunctus*.[8] During our collection, *A. limosus* was not, however, found, thus necessitating future collections of mollusks in other endemic areas of Ecuador.[3,4] and from other regions and countries where *Amphimerus* have been reported in biliary ducts of birds and mammals.[22–24]

The genus *Aroapyrgus* has also been incriminated as the first intermediate host of the lung fluke *Paragonimus mexicanus* in Central and South America.[12,25] In Ecuador, *Aroapyrgus* has been found to shed cercariae of *P. mexicanus* in the Amazon region.[26] The presence of *Aroapyrgus* in the Amazon basin would imply a probable transmission of *Amphimerus* in this jungle area of South America, as has been demonstrated in sylvatic animals from Brazil, Colombia, and Peru.[22–24] Because the tropical area surveyed in this study is also considered endemic for paragonimiasis[27] a molecular differentiation between these two flukes was essential; DNA from a worm isolated from the Ecuadorian human case, when tested for *P. mexicanus* by our RT-PCR, was negative. That result demonstrated that the cercariae isolated from the *Aroapyrgus* corresponded to only *Amphimerus*, verifying also that no coinfection with *P. mexicanus* had occurred. In addition, the primers designed here differentiated this species entirely from the other liver flukes *C. sinensis*, which species is also a member of the Opisthorchiidae family, and *F. hepatica*, it likewise being present in Ecuador.[28]

The RT-PCR technique, as well as the primers and probe designed for this study, proved to be highly sensitive and

specific for the detection of *Amphimerus* in the adult stage, as isolated cercariae, and even when present in the soft tissue of the snails. The ITS2 region provided conclusive information for the morphotype-3 cercaria encountered in only *Aroapyrgus*, thus indicating that this morphotype pertains to *Amphimerus*. Molecular-genetic techniques are preferable for the correct identification of these organisms during the larval stages, in which a diagnosis based on morphology is difficult because of the lack of specific characteristics.[29]

Of the other three species of snails found, *M. tuberculata* released cercariae of trematodes morphotypes 1 and 2 that did not correspond to *Amphimerus* and for any of the other trematodes tested. The other two species—*B. cousini* and *A. marmorata*—did not shed any type of cercariae. Therefore, future surveys are necessary, both here in Ecuador and in the neighboring countries of Peru, Colombia, and Brazil to collect and test more snail species to identify the other types of cercariae that are known to be present in *M. tuberculata*.[30–32] Studies carried out in those countries also did not identify *Amphimerus* sp. in *M. tuberculata*.

We need to stress that with this research we were able to identify the last epidemiological link that was missing to complete the life cycle of *Amphimerus* in Ecuador. Our previous studies demonstrated that the parasite infected domestic animals such as cats and dogs, as well as humans, as definitive and reservoir hosts.[3–5] In subsequent studies, we also found that the fish *Rhoadsia altipinna*, *Bryconamericus bucay*, *Andinoacara rivulatus*, and *Piabucina aureoguttata* acted as second intermediate hosts, transporting metacercariae, the infectious stage of *Amphimerus* to the definitive hosts, including man.[6] Thus, with the present study we have closed the biologic cycle of this bile-duct trematode. That *Amphimerus* would represent public health threat in the tropical study region of Ecuador, and certainly in other countries and regions around the world where the parasite has been found, is highly likely. Prospective studies such as the present one carried out in Ecuador are necessary for demonstrating the true geographic distribution of potential infection in humans along with the animals involved in disease transmission.

In conclusion, to the best of our knowledge, the results reported here constitute the evidence to determine snails of the genus *Aroapyrgus* to be the first intermediate hosts for *Amphimerus* sp. infecting humans in Ecuador. The life cycle of *Amphimerus* is like that of most members of the family Opisthorchiidae in which freshwater snails and fish serve as primary and secondary intermediate hosts, respectively. Furthermore, the large size of the population of *Aroapyrgus* and the high prevalence with *Amphimerus* infection encountered in this survey might be responsible for the high prevalence of human infection in this area, and thus the study region constitutes a zone of high risk for animal and human infection. Future investigations must be considered to implement effective control strategies. The healthcare authorities are hereby notified of the urgency in carrying out an intervention involving control and elimination strategies since these snails and fish are already known to be involved in the transmission of *Amphimerus*. Accordingly, the lack of sanitation that releases major amounts of egg-laden stools to contaminate the streams and infect the snails and fish needs to be rectified and preventive measures against the spread of the pathogen instigated.

Received June 19, 2021. Accepted for publication August 22, 2021.

Published online October 25, 2021.

Note: Supplemental materials appear at www.ajtmh.org.

Acknowledgments: We thank Donald F. Haggerty, a retired academic career investigator and native English speaker, for editing the final version of the manuscript.

Financial support: This research was supported by a grant from the Direccion General de Investigacion y Vinculacion (DGIV) de la Universidad de las Américas (UDLA), Quito-Ecuador (BIO.CBC.20.02) and by the Japan Agency for Medical Research and Development (AMED 21fk0108136j0102) to HS.

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